Model for the Anaerobic Growth of *Aeromonas hydrophila* K144

ABSTRACT

The combined effects of temperature (5 to 42°C), NaCl (0.5 to 4.5%), pH (5.3 to 7.3), and $NaNO_2$ (0 to 200 μg/ml) on the anaerobic growth of *Aeromonas hydrophila* K144 were studied in brain heart infusion broth using a modified central composite design. Variable combinations were tested in triplicate anaerobic flasks (nitrogen atmosphere in sealed trypsinizing flasks); viable cell counts were made at intervals during incubation by surface plating on tryptic soy agar. Growth curves were generated using the Gompertz equation in conjunction with a nonlinear regression analysis program. Values for the four Gompertz parameters (A, C, B, and M) were obtained for the variable combinations tested. Using response surface techniques, regressions were performed on Ln (B), Ln (M), Sqrt (B), and Sqrt (1/M); quadratic and cubic equations containing the four variables of temperature, NaCl, pH, and $NaNO_2$ were developed to yield predictive values for the B and M Gompertz values. Goodness of fit evaluation of the models was determined by R^2 values. Comparison of predicted and observed values of B and M and evaluation of predicted lag and generation times indicated that the quadratic model gave the best fit. Overall, the variable combinations interacted to affect the generation and lag times. The results indicate that pH, salt level, and nitrite level can be manipulated to decrease the growth of *A. hydrophila* when combined with low temperature incubation and anaerobic conditions.

Organisms of the *Aeromonas hydrophila* group (motile aeromonads, *A. hydrophila*, mesophilic *Aeromonas*) have received recent recognition as foodborne pathogens of concern (*1*). The organism occurs widely in the environment, particularly in various water supplies (*7*). *A. hydrophila* also has been isolated from different foods, including vegetables (*4*) and foods of animal origin (*11*). In the food survey of Palumbo et al. (*11*), *A. hydrophila* was detected in virtually every sample of fish and seafood, red meat, and poultry examined. In addition to its detection in every sample, the organism grew during one week's storage at 5°C.

Consumers are currently demanding foods which are fresher, i.e., given less processing and containing fewer additives. This places increased emphasis on refrigeration as the primary means of restricting the growth of foodborne pathogens as well as spoilage microorganisms. However, *A. hydrophila* is one of a group of foodborne pathogens (*9*) that can grow readily in foods held at 5°C. Thus, inhibition of various foodborne pathogens must depend on the interaction of factors such as NaCl level, pH, and $NaNO_2$ level along with low temperature and anaerobic atmosphere (vacuum packaging). This multifactorial approach to the study of growth kinetics and inhibition has proven useful with organisms such as *Shigella flexneri* (L. Zaika et al., in prep), *Listeria monocytogenes* (*2,3*), *Clostridium botulinum* (*5*), and *Salmonella* (*6*).

The purpose of this study was to investigate the combined effects and interactions of temperature, pH, NaCl level, and $NaNO_2$ level on the kinetics of anaerobic growth of *A. hydrophila* in brain heart infusion (BHI, Difco Laboratories, Detroit, MI) broth with the goal of developing a model that could be used to predict the response of the organism to any combination of variables. This work extends earlier studies on the influence of various factors on the aerobic growth of the organism (*10,12*) and compliments the recently completed aerobic model (*13*).

MATERIALS AND METHODS

Organism

Aeromonas hydrophila K144 was used throughout these studies. All experiments were inoculated from a starter flask; the starter flask was prepared by inoculating 50 ml of BHI broth in a 250-ml flask and incubating overnight (18 to 20 h) at 28°C. Dilutions of the starter flask (made in 0.1% peptone water) were used to inoculate the experimental flasks. The count determined as described below at zero time of incubation was ca. 2×10^3 CFU/ml for all individual experiments.

Culture conditions

The culture medium used was BHI broth. This was modified by the addition of NaCl or $NaNO_2$ (filter sterilized) or by adjustment of pH (with HCl). The basal medium contained 0.5% NaCl and is pH 7.3. Incubation was anaerobic in triplicate trypsinizing flasks (Bellco Glass, Inc., Vineland, NJ) (50 ml of broth of the specific variable combination per 250-ml flask); anaerobic atmosphere was attained by gassing the flasks with nitrogen and then sealing them with a rubber stopper (top) and a rubber septum (side

arm). The flask were shaken (150 rpm) at different experimental temperatures.

Variables and experimental design

The following variables were studied in conjunction with a modified central composite design (*8*): temperature (42, 37, 28, 19, 12, and 5°C); pH (7.3 to 5.3, in 0.5-pH unit increments), NaCl (0.5 to 4.5%, in 1% increments), and $NaNO_2$ (0 to 200 μg/ml in 50-μg increments).

Bacteriology

At appropriate intervals during incubation, aliquots were removed with a syringe and a 23-gauge needle through the rubber septum on the side arm. These samples were then diluted as needed in 0.1% peptone water, and surface plated with a Spiral plater (Model D, Spiral Systems, Bethesda, MD) onto tryptic soy agar (Difco). Colonies were counted with a Laser Counting System (Spiral Systems) after 24 to 36 h incubation at 28°C.

Certain variable combinations did not support growth. No growth is defined as a count at or below the starting count for a period of time appropriate to the variable combination. In most instances where no growth was observed, the count decreased to <21/ml (the lower limit of detection) and remained there.

Data processing

Viable cell counts were converted to $\log_{10}$ and the growth data were fitted by the Gompertz equation (*5*) utilizing ABACUS, an iterative nonlinear regression program (W. Damert, Eastern Regional Research Center, USDA personal communication). The Gompertz equation along with the derived growth kinetics equations are shown in Table 1.

Equation development

Quadratic and cubic polynomial models in temperature, pH, sodium chloride level, and sodium nitrite concentration were generated for Gompertz B and M values using the SAS general linear model procedure. Regressions were performed on Ln (M) and Ln (B), with Ln (B) = missing, when B=O; regressions were also performed on Sqrt (B) and Sqrt (l/M). Thus, four sets of polynomial equations (Ln quadratic (Ln (B) and Ln (M) [LnQuad], Ln cubic (Ln (B) and Ln (M) [LnCubic], square root quadratic (Sqrt(B) and Sqrt (1/M)) [Sqrtquad], and square root cubic (Sqrt(B) and Sqrt(l/M)) [Sqrtcub]) were obtained and evaluated for fit to the observed data.

TABLE 1. *Equations for Gompertz parameters and derived growth kinetics values.*

The Gompertz equation is:

$L(t) = A + C \exp\{-\exp(-B(t-M))\}$, where:

$L(t) = \text{Log}_{10}$ count of bacteria at time (in h) t, [$\log_{10}$(CFU/ml)].

A = asymptotic log count of bacteria as time decreases indefinitely (initial level of bacteria, $\log_{10}$CFU/ml).

C = asymptotic amount of growth that occurs as t increases indefinitely [number of log cycles of growth, $\log_{10}$(CFU/ml)].

B = relative growth rate at M, [$\log_{10}$(CFU/ml)/h] where

M = the time at which the absolute growth rate is maximal (h).

Derived growth kinetics equations;

exponential growth rate (EGR) = B*C/e	[$\log_{10}$(CFU/ml)/h]
generation time (GT) = $\log_{10}2$*e/B*C	h
lag phase duration (lag) = M-1/B	h
Maximum population density (MPD) = A+C	$\log_{10}$(CFU/ml)

RESULTS AND DISCUSSION

In this study, we have evaluated the effect of culture conditions (temperature, pH, NaCl, and $NaNO_2$) on the kinetics of anaerobic growth of *A. hydrophila*. To evaluate growth kinetics, we employed the Gompertz equation (Table 1). Based on previous observations in which the initial counts (Gompertz A parameter) were shown not to influence lag and generation times (*13*) as well as considerations discussed by Buchanan et al. (*2,3*) and Gibson et al. (*5,6*), model development concentrated on the Gompertz B and M parameters.

In addition to developing predictive models relating the influence of temperature, pH, NaCl, and $NaNO_2$ on the anaerobic growth of *A. hydrophila*, we also were able to compare conditions that did not support anaerobic growth (Table 2) with those which did not support aerobic growth (*13*). With the exception of the 42°C variable combination (last data line, Table 2), the other combination which restricted anaerobic growth but permitted aerobic growth was 50 μg/ml or greater $NaNO_2$ and pH below 6.0 combined with 1.5% NaCl or above. This increased sensitivity of *A. hydrophila* to $NaNO_2$ under anaerobic conditions is similar to that reported for *Staphylococcus aureus* (*15*). Nongrowth at 42°C was not anticipated, though 42°C is very close to the maximum temperature at which the organism will grow. Under aerobic conditions, the lag and generation times are 32.48 and 1.21 h, respectively (*13*).

TABLE 2. *Conditions of temperature, NaCl, pH, and $NaNO_2$ which did not support the anaerobic growth of* A. hydrophila.

Temp, °C	% NaCl	pH	$NaNO_2$ mg/L
5	0.5	5.8	200
5	3.5	5.3	0
5	3.5	5.8	150
12	3.5	5.8	50**
12	3.5	5.3	150
19	4.5	6.3	100
19	2.5	5.3	100
19	2.5	5.8	100**
28	3.5	5.8	50**
28	1.5	5.8	150**
37	0.5	5.8	200
37	4.5	5.8	200
37	4.5	5.8	0
37	0.5	5.8	200
37	0.5	5.3	0
37	1.5	5.3	0
37	0.5	5.8	50
37	0.5	5.8	100
37	0.5	5.8	150
42	2.5	6.3	100
42	0.5	7.3	0**
42	0.5	5.3	0
42	1.5	5.3	0

*See text for definition of no growth.
**Grew in this variable combination aerobically but not anaerobically.

The variable combinations studied anaerobically and used to derive the models evaluated represented 61 individual experimental combinations (Table 3). The results

TABLE 3. *Effect of culture conditions on the observed values of the Gompertz parameters B and M and on the generation time (GT) and lag time (lag) for the anaerobic growth of* A. hydrophila *(calculated from actual data by the Gompertz equation).*

Variable	Culture variables				No. of	Calculated				
set	temp,°C	pH	NaCl	$NaNO_2$	replicates	B	M	GT, h	Lag, h	MPD
1	5	7.3	0.5	0	3	.0266	59.0	4.32	21.27	10.57
2	5	7.3	0.5	200	1	.0286	98.0	4.08	63.03	10.71
3	5	6.8	0.5	0	3	.021	80.2	5.77	32.29	10.23
4	5	6.3	0.5	50	1	.0173	98.9	6.47	41.10	10.60
5	5	6.3	0.5	100	1	.0128	120.2	12.15	42.08	8.58
6	5	6.3	0.5	200	1	.00937	293.47	12.98	186.75	10.40
7	5	6.3	1.5	0	1	.0246	69.6	4.88	28.95	10.07
8	5	6.3	1.5	50	1	.0172	105.1	6.30	46.90	10.84
9	5	6.3	1.5	100	1	.200	93.5	0.54	88.5	10.64
10	5	5.8	0.5	0	3	.00497	282.0	24.77	76.86	10.11
11	5	5.3	0.5	0	1	.157	14.5	2.15	167.0	11.00
12	5	5.3	1.5	0	1	.0117	226.5	9.09	141.0	10.85
13	12	7.3	0.5	0	3	.115	37.4	1.0	28.69	11.02
14	12	7.3	0.5	200	1	.134	27.36	0.9	19.80	10.27
15	12	7.3	2.5	0	1	.048	52.30	2.50	31.55	10.47
16	12	7.3	2.5	50	1	.0554	56.70	2.00	38.65	10.48
17	12	7.3	2.5	100	1	.0503	60.0	2.36	40.12	10.60
18	12	7.3	2.5	200	1	.0534	60.6	2.31	41.87	10.48
19	12	7.3	3.5	0	1	.0228	99.4	4.85	55.54	10.56
20	12	7.3	3.5	50	1	.0207	97.6	5.04	49.29	10.66
21	12	7.3	3.5	100	1	.0210	102.1	5.01	54.48	10.66
22	12	7.3	3.5	200	1	.0201	90.4	5.06	40.65	10.69
23	12	6.8	1.5	50	6	.046	32.0	2.82	10.07	11.06
24	12	6.8	1.5	150	3	.043	42.8	2.58	19.35	10.65
25	12	6.8	3.5	50	3	.028	87.67	7.60	51.77	7.58
26	12	6.8	3.5	150	3	.0319	79.0	6.23	47.38	8.07
27	12	6.3	2.5	100	3	.0084	480.8	16.53	35.46	10.12
28	12	5.8	1.5	50	3	.0264	150.5	4.9	111.38	10.22
29	12	5.3	1.5	0	1	.093	72.9	1.67	62.15	9.16
30	19	7.3	0.5	0	3	.138	13.1	0.77	5.82	11.19
31	19	7.3	2.5	100	3	.203	31.6	0.59	26.71	10.56
32	19	7.3	0.5	200	1	.158	14.21	0.71	7.88	10.76
33	19	6.3	0.5	0	1	.155	16.20	0.75	9.75	10.58
34	19	6.3	0.5	100	3	.173	24.8	0.76	19.93	10.38
35	19	6.3	2.5	0	3	.206	28.5	0.63	23.14	10.73
36	19	6.3	2.5	100	3	.050	42.13	2.50	22.0	10.26
37	19	6.3	3.5	0	1	.0901	34.4	1.36	23.30	10.17
38	19	6.3	2.5	200	3	.100	29.2	1.03	19.25	10.95
39	19	5.3	0.5	0	1	.116	31.1	0.99	22.48	10.68
40	19	5.3	1.5	0	1	.0878	32.4	1.35	21.0	10.87
41	28	7.3	0.5	0	3	.336	6.59	0.37	3.61	10.14
42	28	7.3	0.5	200	1	.251	7.59	0.47	3.61	10.70
43	28	7.3	3.5	0	1	.343	22.11	0.33	19.19	11.03
44	28	6.8	1.5	50	6	.181	8.36	0.62	2.78	11.00
45	28	6.8	1.5	150	2	.167	96.40	0.75	3.54	10.97
46	28	6.8	3.5	50	3	.267	16.20	0.55	12.43	10.99
47	28	6.8	3.5	150	6	.197	17.4	0.65	11.87	10.32
48	28	5.8	1.5	50	3	.153	19.4	0.83	12.48	10.40
49	28	5.8	3.5	150	3	.143	28.2	1.03	21.24	9.25
50	28	5.8	3.5	200	1	.345	19.41	0.32	16.51	11.33
51	28	5.3	0.5	0	1	.170	13.5	0.70	7.62	10.44
52	28	5.3	1.5	0	1	.157	14.5	0.75	8.13	10.87
53	37	7.3	0.5	0	2	.366	4.85	0.40	2.11	9.67
54	37	7.3	0.5	100	1	.244	6.8	0.58	2.70	9.50
55	37	7.3	0.5	200	1	.180	8.0	0.81	2.44	9.50
56	37	6.3	0.5	0	1	.251	6.22	0.52	2.24	9.70
57	37	6.3	0.5	50	1	.207	6.90	0.62	2.07	9.62
58	37	5.8	0.5	0	3	.285	7.31	0.78	3.12	7.92
59	37	5.8	0.5	25	1	.165	11.56	0.80	5.50	9.72
60	37	5.8	1.5	0	1	.186	9.04	0.76	3.66	9.42
61	37	5.8	2.5	0	1	.0840	56.81	2.74	44.91	7.97

TABLE 4. *Predicted lag and generation time (GT) (both in hours) for the anaerobic growth of* A. hydrophila *calculated from values for B and M obtained from the Ln quadratic (LnQuad), Ln cubic (LnCubic), square root quadratic (Sqrtquad), and square root cubic (sqrtcubic) polynomial equations.*

Variable	LnQuad		LnCubic		Sqrtquad		Sqrtcubic	
set	Lag	GT	Lag	GT	Lag	GT	Lag	GT
1	28.41	4.80	45.77	1.98	32.42	10.38	44.13	3.81
2	71.0	4.18	30.22	1.51	-251.37	74.05	26.99	0.70
3	26.18	6.19	53.34	3.33	28.42	4.40	6.75	5.99
4	69.80	8.26	84.16	6.24	210.06	18.25	65.61	5.71
5	103.22	8.49	103.62	2.69	203181	2388.68	102.11	6.67
6	150.41	7.22	125.17	5.87	-209.76	73.49	541.26	3.89
7	56.98	10.54	77.27	3.46	78.53	8.48	40.11	13.78
8	93.18	11.72	90.57	4.67	1158.57	125.67	-8.88	15.96
9	129.39	12.12	133.08	3.01	9.68	214.25	63.05	20.26
10	80.58	8.44	130.10	12.99	64.83	3.78	119.30	6.68
11	184.11	8.92	137.16	3.88	387.83	6.68	74.30	5.73
12	265.47	12.99	222.17	3.92	9766.93	60.17	42.15	2.64
13	12.17	1.50	24.62	0.93	8.84	1.09	15.85	1.53
14	22.38	1.42	11.78	0.77	9.61	1.63	19.15	0.67
15	30.96	2.43	47.47	1.77	49.85	2.27	70.06	6.52
16	36.17	2.71	40.11	1.41	56.18	2.91	30.63	3.13
17	38.30	2.81	38.50	0.69	43.77	2.72	24.81	2.20
18	32.00	2.41	32.16	1.86	14.15	1.14	-962.65	128.31
19	54.69	3.29	63.50	2.66	410.92	5.67	63.69	3.73
20	59.87	3.68	55.48	2.63	318.17	6.27	26.12	3.55
21	59.48	3.84	50.24	1.23	130.03	4.42	20.41	3.37
22	43.67	3.35	21.41	1.42	18.17	1.19	-6135.97	776.31
23	24.10	2.71	14.98	1.06	19.01	1.79	11.71	2.88
24	31.95	2.74	16.30	1.08	24.44	2.32	0.61	4.48
25	63.24	4.85	66.49	2.81	139.46	6.85	23.07	6.59
26	62.39	5.02	44.67	2.82	55.13	3.78	-74.31	17.07
27	60.82	4.63	151.88	3.51	75.50	5.84	31.56	10.51
28	59.94	3.79	100.76	2.39	55.89	3.51	285.39	14.31
29	84.94	3.47	78.32	2.40	42.67	2.40	55.22	2.52
30	6.12	0.67	8.54	0.41	4.92	0.56	4.98	0.65
31	17.71	1.04	19.81	0.44	13.92	0.90	16.80	1.11
32	8.79	0.69	3.03	0.40	4.82	0.71	6.39	0.50
33	6.56	1.02	12.79	1.10	5.10	0.50	3.45	0.55
34	12.69	1.26	13.20	0.52	12.41	1.20	11.58	1.40
35	18.82	1.34	21.04	0.44	11.33	0.88	14.20	0.66
36	25.49	1.70	39.50	1.54	21.49	1.63	17.93	2.74
37	33.52	1.63	22.08	0.66	23.00	1.59	31.96	0.86
38	23.40	1.62	8.73	0.92	17.96	1.26	-91.90	14.75
39	23.50	1.19	23.47	0.77	13.41	0.83	17.71	1.43
40	34.55	1.35	21.73	1.27	18.75	1.24	19.67	1.41
41	3.37	0.39	2.08	0.21	4.11	0.48	2.29	0.41
42	3.40	0.46	0.02	0.28	3.52	0.58	1.49	0.44
43	15.52	0.40	14.87	0.16	29.44	0.83	19.64	0.26
44	5.58	0.57	2.31	0.37	7.36	0.67	2.41	0.58
45	5.40	0.64	1.55	0.39	7.54	0.76	-0.61	1.06
46	15.53	0.61	10.71	0.30	22.23	1.03	10.49	0.49
47	12.48	0.70	5.86	0.36	12.81	0.77	6.46	0.99
48	10.79	0.78	9.54	0.65	18.04	1.47	8.08	1.82
49	27.33	1.03	13.65	0.60	111.82	6.36	-130.06	29.77
50	23.47	1.01	1.73	0.05	72.48	3.96	-7.96	4.64
51	10.19	0.69	5.92	0.30	13.67	1.00	6.96	0.89
52	15.54	0.68	5.56	0.70	19.81	1.43	7.97	1.05
53	2.46	0.42	0.76	0.27	5.59	0.81	2.89	0.84
54	2.30	0.55	1.16	0.24	5.10	1.34	2.09	0.87
55	1.10	0.54	-2.10	0.58	3.45	0.99	-5.04	1.33
56	1.23	0.62	1.37	0.24	6.80	1.04	1.30	0.37
57	1.43	0.76	0.66	0.52	10.01	2.07	-0.27	1.04
58	2.42	0.69	2.13	0.42	11.91	1.68	3.06	0.56
59	2.81	0.77	3.15	0.47	18.08	2.90	3.13	1.53
60	5.62	0.58	5.80	0.54	21.70	2.21	6.17	0.67
61	10.17	0.51	28.56	0.97	52.59	3.84	12.64	0.70

*See Table 3 for the temperature, pH, NaCI and $NaNO_2$ combination corresponding to each variable set.

consisted of 118 individual growth curves. These were analyzed for the Gompertz B and M parameters as well as the derived kinetic parameters of lag and generation times and maximum population density (MPD) (Table 3); the average MPD for all experiments in which growth occurred was 10.19. The anaerobic growth kinetics in Table 3 were in general agreement with those of the aerobic study (*13*): both generation and lag times increased with decreasing temperature and pH and increasing salt and nitrite levels. Overall, except for the conditions under which the organism did not grow anaerobically compared to aerobically, anaerobic conditions did not restrict the organism's growth kinetics, i.e., *A. hydrophila* grew as well anaerobically as it did aerobically. Though the organism is considered to be a facultative anaerobic (*14*), the observed kinetics were not affected by the anaerobic (nitrogen) atmosphere used in these studies.

To select the appropriate polynomial model which best described the organism's anaerobic response to variations in temperature, pH, NaCl, and sodium nitrite, several different approaches were used. In the first, predicted values for generation and lag times for each variable combination using each set of polynomial equations were calculated (Table 4). These predicted generation and lag times can then be compared to the observed values in Table 3. Some generalizations of the data in Table 4 can be made. For certain variable combinations, the square root models (both quadratic and cubic) predicted negative lag times, some very large lag times, and some very large generation times. In addition, the LnCubic model yielded one negative lag time (variable set 55) and one very short GT (variable set 50). Based on these considerations and those presented below, it was concluded that the LnQuad equations for B and M (Table 5) best describe the influence of the four variable combination on the anaerobic growth of *A. hydrophila*.

TABLE 5. *Second order (quadratic) polynomial response surface model in temperature (°C), pH, NaCl (%), and $NaNO_2$ (mg/L) for the Gompertz parameters B and M for anaerobic growth of* A. hydrophila.

$$\text{Ln (B)} = -1.6765 + 0.22881*\text{temp} - 1.351*\text{pH} - 0.4402*\text{NaCl} - 0.0057*\text{NaNO}_2 - 0.00128*\text{temp}*\text{pH} + 0.01616*\text{temp}*\text{NaCl} - 0.000063*\text{temp}*\text{NaNO}_2 + 0.0088*\text{pH}*\text{NaCl} + 0.000531*\text{pH}*\text{NaNO}_2 - 0.000119*\text{NaCl}*\text{NaNO}_2 - 0.0036*\text{temp}*\text{temp} + 0.1319*\text{pH}*\text{pH} - 0.0198*\text{NaCl}*\text{NaCl} + 0.00001452*\text{NaNO}_2*\text{NaNO}_2$$

$$\text{Ln (M)} = 20.9965 - 0.2637*\text{temp} - 4.241*\text{pH} + 0.3282*\text{NaCl} + 0.0175*\text{NaNO}_2 + 0.01*\text{temp}*\text{pH} - 0.00324*\text{temp}*\text{NaCl} - 0.0000792*\text{temp}*\text{NaNO}_2 - 0.00392*\text{pH}*\text{NaCl} - 0.0015*\text{pH}*\text{NaNO}_2 - 0.00787*\text{NaCl}*\text{NaNO}_2 + 0.00276*\text{temp}*\text{temp} + 0.27965*\text{pH}*\text{pH} + 0.03548*\text{NaCl}*\text{NaCl} - 0.0000177*\text{NaNO}_2*\text{NaNO}_2$$

A second means of evaluating the models generated for their ability to describe the influence of the anaerobic growth variables on *A. hydrophila* is shown in Table 6. The R^2 values for B and M in ascending order are Sqrtquad, Quad, Sqrtcub, and Cubic. The closer the R^2 values to 1.0, the better the derived models fit the data used to generate them. Thus, on the basis of R^2 values, the cubic polynomial models best fit the observed growth kinetics of the organism.

TABLE 6. *Comparison of R^2 values for the four predictive models generated for B and M.*

Model	Gompertz parameter B	Gompertz parameter M
LnQuadratic	0.7813*	0.8721
LnCubic	0.8665	0.9558
Sqrtquad	0.6615	0.7091
Sqrtcub	0.8183	0.8816

*R^2 = Square of multiple correlation coefficient; percentage of variability in the response accounted for by the model.

The third means of evaluating the models is our so-called "use" test. In this procedure, variable combinations not used to generate the original four predictive models were inserted into the respective equations and the corresponding lag and generation times generated (Table 7). Using this procedure, only the LnQuad model yielded realistic estimates; the other three models (data not shown) gave negative lag and generation times (GTs), some very large GTs, and some very short GTs. Thus, based on the "use" test, the LnQuad model gave the "best" estimates for GT and lag. In our aerobic study (*13*), the LnCubic model also yielded this same sort of "nonsense" values. In this study, we expanded the variable combinations incorporated into the models, particularly in these areas of the data base, to avoid generation of these "nonsense" values, but our expanded data base did not avoid this problem.

TABLE 7. *Comparison of lag and GT predicted from LnQuad model for various variable combinations.*

Variables				Model	
Temp, °C	pH	NaCl	$NaNO_2$	LnQuad Lag	LnQuad GT
5	6.0	1.0	100	166.96	11.13
5	6.0	3.0	0	181.59	20.74
5	5.5	1.0	50	249.15	11.81
10	7.0	3.0	50	58.36	5.41
10	6.5	2.0	50	45.20	4.94
10	5.5	3.0	25	233.28	8.99
20	7.0	3.0	50	20.27	1.12
20	6.5	2.0	50	14.37	1.20
20	5.5	3.0	25	54.95	1.69
30	7.0	3.0	50	10.79	0.48
30	6.0	2.0	50	9.76	0.69
30	5.5	2.5	25	19.26	0.69
35	7.0	3.0	50	9.44	0.41
35	6.0	2.0	50	7.75	0.64
35	5.5	1.0	100	9.69	0.90

In summary, this study presents one of the first evaluations of the influence of temperature, pH, NaCl, and $NaNO_2$ on the anaerobic growth of *A. hydrophila*. Overall, the growth kinetics are similarly affected by these culture variables under anaerobic (nitrogen atmosphere) conditions as they were under aerobic conditions. However, as can be seen in Table 2, there are certain culture variable combinations which did not support anaerobic growth, but which did support aerobic growth of the organism. Again, as with our aerobic study (*13*), the Quad polynomial equations (Table 5) were concluded to be best. This is based primarily on the "use" test (Table 7) and comparison of observed lag and GTs (Table 3) with those predicted by the different models generated (Table 4).

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